FGFR-2 and -3 Play an Important Role in Initial Stages of Oral Oncogenesis

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Abstract. *Background: FGFR-2 and FGFR-3 (fibroblast growth factor receptors) have been shown to play an important role in several processes including carcinogenesis. This study was designed to determine gradual FGFR-2 and FGFR-3 expression in sequential stages of oral carcinogenesis in an experimental animal system of Syrian golden hamsters. Materials and Methods: Tissue sections ranging from normal mucosa to squamous cell carcinoma were studied using monoclonal antibodies against FGFR-2 and FGFR-3 proteins. Results: A significant elevation was revealed in both FGFR-2 and FGFR-3 expression during the stages of dysplasia and early invasion, while in the later stages of oral carcinogenesis the expression of both FGFR-2 and FGFR-3 decreased although not significantly. Conclusion: Our findings indicate that FGFR-2 and FGFR-3 seem to play an important role in the initial stages of oral cancer progression.*

Oral squamous cell carcinoma (OSCC) is considered to be the sixth most common malignancy worldwide and is associated with severe morbidity and high mortality (1). More than 300,000 new cases of OSCC are being diagnosed annually all over the world (2). Nevertheless, the results of the treatment of oral cancer patients have not shown any improvement during the last decades, despite advances in treatment with surgery, radiation and chemotherapy (3). It is widely accepted that cancer arises as a result of the accumulation of genetic alterations (1). The term 'oncogenes' belongs to genes whose protein products have been found to be important for normal cell growth and whose mutation or overexpression results in uncontrolled cell growth and carcinogenesis (1). On the other hand, tumour suppressor genes are involved in cell cycle regulation, including cell cycle arrest and apoptosis and their loss of function requires alteration of both alleles (1).

Fibroblast growth factor receptors (FGFRs) are transmembrane proteins with tyrosine kinase activity. Multiple signal transduction cascades are initiated after fibroblast growth factor (FGF) ligand binding to the extracellular domain of the receptor, which ultimately results in modification of gene expression (4). The best understood of these are the RAS-MAP kinase pathway which include ERK1/2, p38 and JNK kinases; the P-I-3 kinase-AKT pathway and the PLCγ pathway (5).

There are four FGFR genes which have 55-72% amino acid homology, FGFR-1(flg), FGFR-2 (bek), FGFR-3 and FGFR-4, encoding several splice-variants that increase the signalling repertoire (6, 7). FGFRs have been shown to play an important role in several processes of embryonic development and tissue homeostasis and their abnormal expression or mutation can cause diverse pathologies ranging from morphogenetic disorders to cancer (4).

Since there is scarce literature concerning FGFR expression in OSCC, our study was designed to determine the expression of two of these receptors, FGFR-2 and FGFR-3, and their possible correlation in sequential stages of OSCC

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formation in an experimental animal system of induced oral carcinogenesis. Syrian golden hamsters were used as in our previous study investigating p53 and c-myc protein expression (8). The chemically induced oral premalignant lesions and OSCC resemble both pathologically and ultrastructurally the equivalent ones that develop in humans exposed to tobacco and alcohol (9, 10).

Materials and Methods

Experimental carcinogenesis. Thirty-seven male Syrian golden hamsters (*Mesocricetus auratus*) purchased from the Hellenic Pasteur Institute (Athens, Greece) at the age of five weeks and weighing approximately 100 g each, were used in this study. The hamsters were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animals were randomly divided into four groups: one control (n=7) and three experimental groups for carcinogen treatment (A, B and C; n=10 animals each). The left buccal pouches of animals in experimental groups were treated with 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA) (Sigma, St. Louis, MO, USA) dissolved in paraffin oil, for 10 weeks (group A) and for 14 weeks (groups B and C), as previously described (11). The pouches of all animals were examined weekly in order to observe the growth of tumours on the mucosa. The treated buccal pouches were removed after sacrifice of animals at 10 weeks from the first application of the carcinogen (group A), at 14 weeks (group B) and at 19 weeks (group C). The control animals were sacrificed at 10 weeks and a left buccal pouch sample was taken. The biopsies were given a number and examined blindly.

Pathological evaluation. Each section was examined under light microscopy and all possible different lesion types were evaluated. The tissue profiles were classified into totally normal oral mucosa, non-cancerous (hyperkeratosis, hyperplasia), pre-cancerous (dysplasia) and cancerous conditions (early invasion, well- and moderately-differentiated carcinoma).

Immunohistochemical analysis. The biopsies from the 37 animals were fixed in 10% neutralized formaldehyde solution and embedded in paraffin. Three sections of 4 μm were prepared from each specimen and were mounted on Super Frost Plus-coated glass slides (Menzel and Co., Braunschweig, Germany). One section was stained with hematoxylin and eosin (H&E) for routine histological evaluation, while the other two were used for immuno-histochemical detection of FGFR-2 and FGFR-3 proteins. The sections were incubated with monoclonal primary antibodies against FGFR-2 (Bek C-17:sc-122, Santa Cruz Biotechnology, Santa Cruz, CA, USA, diluted 1:200) and FGFR-3 (FGFR-3 C-15:sc-123, Santa Cruz Biotechnology, diluted 1:200), as previously described (11). Oral carcinoma tissue and normal skin which strongly express FGFR-2 and FGFR-3, respectively, were used as positive controls. Negative controls for each biopsy were processed in the same manner, using phosphate-buffered saline (PBS) instead of the primary antibody. All slides were independently reviewed by two investigators blindly. The consecutive H&E-stained slides were evaluated by a pathologist experienced in oral pathology, without knowing the FGFR-2 and FGFR-3 staining patterns.

Table I. *Histological status of biopsies in the control group and the three experimental groups.*

	Control group	Group A	Group B	Group C
Normal tissue	4			
Hyperkeratosis	2			
Hyperplasia	1			
Dysplasia		6		
Early invasion		3	1	
Well-differentiated carcinoma		1	6	1
Moderately-differentiated carcinoma			3	9

Statistical analysis. The mean value of positive stained cells percentages was calculated from all the different lesions present in each sample. These values were tabulated for each group of animals (control group and experimental groups A, B, C) and compared.

In order to evaluate the pattern of antibody expression in relation to the histological status, the various lesions were divided according to tumour progression into: a) normal tissue, b) non-cancerous and pre-cancerous conditions (hyperkeratosis, hyperplasia, dysplasia) and c) tumour (early invasion, well-differentiated carcinoma, moderately-differentiated carcinoma). In every lesion the percentages of positively-stained cells from each non-cancerous and pre-cancerous category were compared with those of the normal tissue, while the percentages of positively-stained cells from each tumour category were compared with the average percentage of the two non-cancerous and one pre-cancerous lesions.

A two-tailed Student's *t*-test was applied for statistical analysis using the SPSS 10.0 program for Windows™. In addition, in every group and every histological category, a normal distribution check was performed using the Kolmogorov-Smirnov Z test of the SPSS. If a group or a histological category was not normally distributed, additional statistical analysis was performed with the Wilcoxon test using the SPSS.

Results

A progression towards OSCC formation in correlation with increased time from initiation of carcinogenesis was evident by the histological status of biopsies in the four studied groups of animals (Table I). Therefore, as expected, this experimental model seemed valid and further analysis of immunostaining data (Figures 1 and 2) was implemented. Interestingly, immunohistochemical staining of tissue sections stromal cells was observed for both antibodies. In all cases with no normal distribution, the results of both the Wilcoxon test and two-tailed Student's *t*-test provided the same level of significance.

The percentages of cells expressing the FGFR-2 and FGFR-3 gene products in the various categories of histological status are shown in Table II. An elevation in both proteins' expression was noted during the stages of dysplasia and early invasion followed by a decrease during

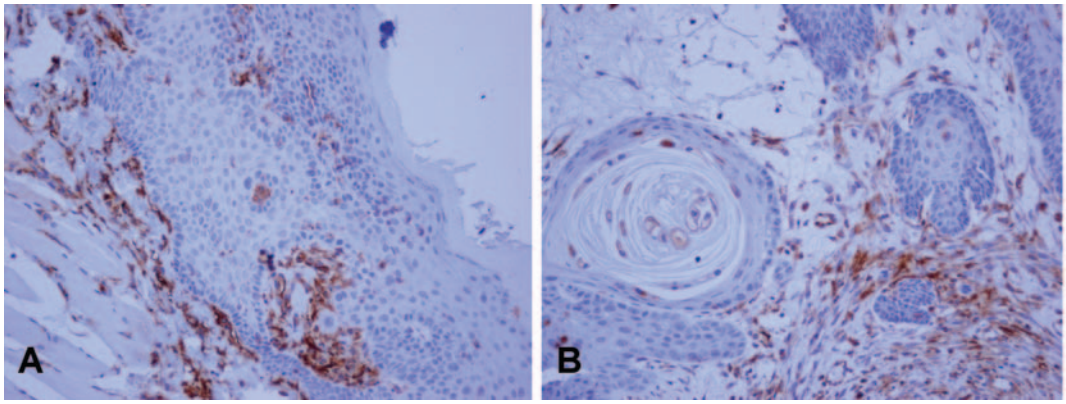

Figure 1. *Immunohistochemical staining of oral tissues using FGFR-2 antibody: (A) FGFR-2 stromal immunoexpression (+) in hyperplastic oral mucosa (x200) (B) Strong FGFR-2 stromal immunoexpression (+) in an area of early invasion (x200).*

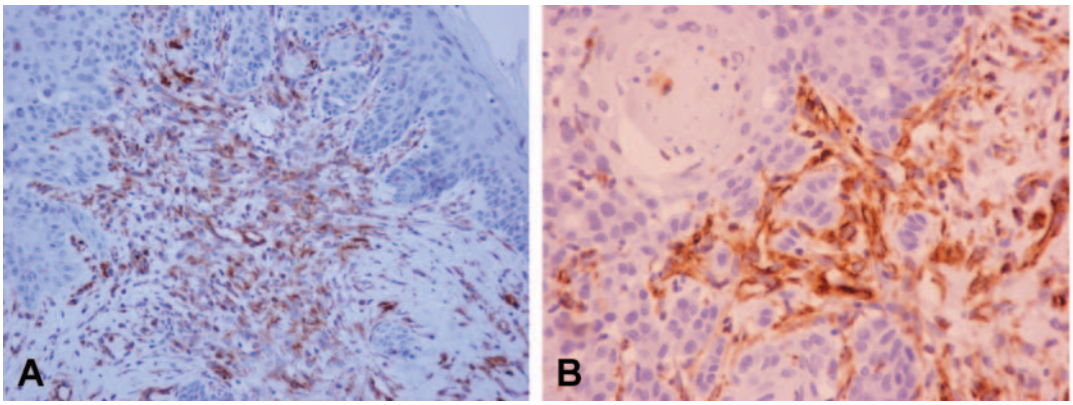

Figure 2. *Immunohistochemical staining of oral tissues using FGFR-3 antibody: (A) FGFR-3 stromal immunoexpression (+) in dysplastic oral mucosa (x200) (B) FGFR-3 stromal immunoexpression (+) in an area of early invasion (x400).*

the following stages of oral carcinogenesis. The statistical analysis of the FGFR-2 revealed statistically significant differences in the dysplastic oral tissues compared to normal oral mucosa ($p<0.05$), as well as in early invasive tissues compared to pre-cancerous tissues ($p<0.001$). Similarly, with the FGFR-3, statistically significant differences were observed in the same stages; in the dysplastic oral tissues compared to normal oral mucosa ($p<0.01$), as well as in early invasive tissues compared to non-cancerous and pre-cancerous tissues ($p<0.001$).

The percentages of cells expressing the FGFR-2 and FGFR-3 gene products in the control group and in the three experimental groups are demonstrated in Table III. The comparison of the four animal groups revealed a statistically significant increase of FGFR-2 only between experimental groups B and A ($p<0.05$) followed by an insignificant decrease between experimental groups C and B. The FGFR-3 analysis of the four groups revealed a statistically significant increase only between experimental group A and the control group ($p<0.05$).

Discussion

In this study, an experimental model of chemically induced tumours of the hamster buccal pouch was used to establish a model for oral carcinogenesis. In this model the expression levels of the oncogenic products of the FGFR-2 and the FGFR-3 were investigated by immunohistochemical procedures in various stages of OSCC formation.

An elevation in both FGFR-2 and FGFR-3 expression during the stages of dysplasia and early invasion was revealed. This increase did not persist, as in the later stages of oral carcinogenesis, both FGFR-2 and FGFR-3 expression decreased again, although not significantly. We have previously demonstrated that H-ras, a downstream effector protein of the MAPK cascade, decreases in the same stages of dysplasia and early invasion (12). Therefore, it can be assumed that a different FGFR-related signaling pathway may be implicated in the pathogenesis of OSCC, such as the P-I-3 kinase-AKT pathway or the PLCγ pathway (4).

Table II. *Percentages of FGFR-2 and FGFR-3 positive cells in the various categories of histological status.*

		Non-cancerous and pre-cancerous			Tumour		
	Normal oral mucosa	Oral mucosa with hyperkeratosis	Oral mucosal hyperplasia	Oral mucosal dysplasia	Early invasion	Well-differentiated OSCC	Moderately-differentiated OSCC
FGFR-2							
Mean value of percentages (%)	(N=15) 0	(N=5) 0	(N=16) 0.06	(N=22) 0.41	(N=19) 2.5.	(N=24) 0.5	(N=25) 0.36
Mean value of percentages of non-cancerous and pre-cancerous conditions (%)			0.23				
Probability of *t*-test		N.S.	N.S.	$p<0.05$	$p<0.001$	N.S.	N.S.
FGFR-3							
Mean value of percentages	(N=15) 0.13	(N=5) 0	(N=16) 0.87	(N=22) 2.0	(N=19) 5.84	(N=24) 1.3	(N=25) 0.72
Mean value of percentages of non-cancerous and pre-cancerous conditions			1.35				
Probability of *t*-test		N.S.	N.S.	$p<0.01$	$p<0.001$	N.S.	N.S.

N.S.: No statistical difference.

This study seems to be the first one performed in an experimental animal model of induced oral carcinogenesis in which the percentages of positively-stained cells were studied in detail during the various stages of oral cancer progression. There are rare data in the literature concerning FGFR-2 and/or FGFR-3 immunoexpression in oral cancer. A study of FGFR-2 and FGFR-3 which was performed in human oral tissue samples during the various stages of oral cancer progression (normal epithelium, mild dysplasia, moderate/severe dysplasia, *in situ* carcinoma and OSCC) has demonstrated a statistically significant increase in staining intensity of FGFR-2 and FGFR-3 in the dysplasias and the squamous cell carcinomas (13).

In addition, one study in human squamous cell carcinoma of the tongue has investigated where fibroblast growth factors were synthesized and stored and where the cells with receptors were located in normal oral mucosa and squamous carcinoma of the tongue (14). This study investigated the localization of fibroblast growth factors and their receptors without analysis of FGFR expression in the various stages.

In conclusion, our findings indicate that FGFR-2 and FGFR-3 seem to play an important role in the initial stages of oral cancer progression. Therefore, taking into consideration the lack of information in the literature concerning their progressive expression in oral cancer, further investigation is required.

Table III. *Percentages of FGFR-2 and FGFR-3 positive cells in the control group and in the three experimental groups.*

	Control Group	Group A	Group B	Group C
FGFR-2				
Mean value of percentages (%)	(N=7) 0	(N=10) 0.47	(N=10) 1.12	(N=10) 0.62
Probability of *t*-test		N.S.	$p<0.05$	N.S.
FGFR-3				
Mean value of percentages (%)	(N=7) 0.3	(N=10) 2.6	(N=10) 2.1	(N=10) 1.6
Probability of *t*-test		$p<0.05$	N.S.	N.S.

N.S.: No statistical difference.

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References

1 Das BR and Nagpal JK: Understanding the biology of oral cancer. Med Sci Monit *8*: 258-267, 2002.

2 Sudbo J: Novel management of oral cancer: a paradigm of predictive oncology. Clin Med Res *2(4)*: 233-242, 2004.

3 Sudbo J, Bryne M, Mao L, Lotan R, Reith A, Kildal W, Davidson B, Soland TM and Lippman SM: Molecular based treatment of oral cancer. Oral Oncol *39*: 749-758, 2003.

4 Dailey L, Ambrosetti D, Mansukhani A and Basilico C: Mechanisms underlying differential responses to FGF signaling. Cytokine Growth Factor Rev *16*: 233-247, 2005.

5 Schlessinger J: Cell signaling by receptor tyrosine kinases. Cell *103*: 211-225, 2000.

6 Givol D and Yayon A: Complexity of FGF receptors; genetic basis for structural diversity and functional specifity. FASEB J *6*: 3362-3369, 1992.

7 Grose R and Dickson C: Fibroblast growth factor signalling in tumourigenesis. Cytokine Growth Factor Rev *16*: 179-186, 2005.

8 Papakosta V, Vairaktaris E, Vylliotis A, Derka S, Nkenke E, Vassiliou S, Lazaris AC, Mourouzis C, Rallis G, Spyridonidou S, Anagnostopoulou S, Perrea D, Donta I, Yapijakis C and Patsouris E: The co-expression of c-myc and p53 increases and reaches a plateau in early stages of oral oncogenesis. Anticancer Res *26(4B)*: 2957-2962, 2006.

9 Chang KW, Saraj S, Lin SC, Tsai PI and Solt D: p53 expression p53 and Ha-ras mutation and telomerase activation during nitrosamine-mediated hamster pouch carcinogenesis. Carcinogenesis *21(7)*: 1441-1451, 2000.

10 Balasenthil S, Saroja M, Ramachandran CR and Nagini S: Humans and hamsters: comparative analysis of lipid peroxidation, glutathione and glutathione-dependent enzymes during oral carcinogenesis. Br J Oral Maxillofac Surg *38*: 267-270, 2000.

11 Derka S, Vairaktaris E, Papakosta V, Vassiliou S, Acil Y, Vylliotis A, Spyridonidou S, Lazaris AC, Mourouzis C, Kokkori A, Moulavassili P, Perrea D, Donta I, Yapijakis C and Patsouris E: Cell proliferation and apoptosis culminate in early stages of oral oncogenesis. Oral Oncol *42(5)*: 540-550, 2006.

12 Vairaktaris E, Papakosta V, Derka S, Vassiliou S, Nkenke E, Spyridonidou S, Vylliotis A, Lazaris AC, Kokkori A, Moulavassili P, Loukeri S, Perrea D, Donta I, Yapijakis C and Patsouris E: H-ras and c-fos exhibit similar expression patterns during oral oncogenesis. Acta Odontol Scand, 2006 (in press).

13 Wakulich C, Jackson-Boeters L, Daley TM and Wysocki GP: Immunohistochemical localization of growth factors fibroblast growth factor-1 and fibroblast growth factor-2 and receptors fibroblast growth factor receptor-2 and fibroblast growth factor receptor-3 in normal oral epithelium, epithelial dysplasias, and squamous cell carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod *93*: 573-579, 2002.

14 Forootan SS, Ke, Y, Jones AS and Helliwell TR: Basic fibroblast growth factor and angiogenesis in squamous cell carcinoma of the tongue. Oral Oncol *36*: 437-443, 2000.

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